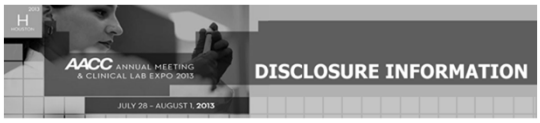
Immunosuppressants

James C. Ritchie, PhD

***September* 17, 2013**



DISCLOSURE STATEMENT

Speaker: James C. Ritchie, Ph.D.

Dr. Ritchie has disclosed the following financial relationships. Any real or apparent conflicts of interest related to the content of this presentation have been resolved.

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- T2 Biosciences
- Chromsystems

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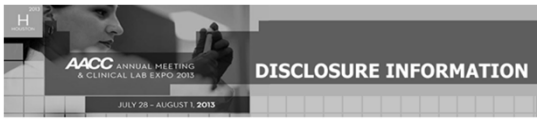
- MH-078105 - Early Experience, Stress and Neurobehavioral Development Center



Unapproved or Off Label Disclosures for James C. Ritchie, PhD

James Ritchie has documented that his presentation involves comments or discussion of a validated Laboratory Developed Test (LDT) employing liquid chromatography and mass spectrometry for use in the clinical laboratory.

Emory Transplant Center

❖**Region's largest and only comprehensive organ and tissue transplant program.**

In 2010 the Emory Center performed a total of 453 transplants:

160 Kidney
22 Heart
80 Liver
26 Pancreas
24 Lung
128 Stem Cell
13 Islet Cell

EMORY HEALTHCARE
EMORY HOSPITALS

Classification of Immunosuppressant Drugs

Category	Types	Example
Immunophilin-binding drugs	Calcineurin inhibitors	**Cyclosporine A, Tacrolimus**
	mTOR inhibitors	**Sirolimus**, Everolimus
Anti-metabolites	Inhibitors of de *novo* purine synthesis	Mycophenolic acid (MPA) Mycophenolate Mofetil (MMF), azathioprine
	Inhibitors of de *novo* pyramidine synthesis	Leflunomide
Biologic immunosuppression	Polyclonal antibodies	Anti-thymocyte gamma globulin thymoglobulin
	Monoclonal antibodies	Anti-CD3 monoclonal antibody (OKT3), IL-2H (humanized), Belatacept, Basiliximab
Others		Deoxyspergualin, corticosteroids, fingolimod (FTY720)

For the ISDs routinely measured today, it is still recommended that methods specific for the parent molecule be used.

2003
Immunosuppressant Testing

Measurand	Volume / yr	Cost / Analysis	Cost / yr
Cyclosporine	7588	$10.80	$81,950
Tacrolimus	13120	$12.00	$157,440
Rapamycin	744	$ 57.45*	$42,743
TOTALS	**21,452**		**$282,133**

*Sendout-Mayo Labs

- Rapamycin TAT = 1 to 4 days
- Expecting volume to increase substantially in future
- Each drug is a separate analysis, no opportunity for multiplexing
- Total cost to system over 7 years = $1,974,931

Immunoassays

Pros

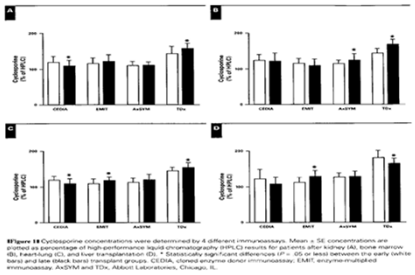
- Very sensitive
- Lab friendly / automated
- Good precision
- FDA approved methods

Cons

- Cross reactivity issues
- Heterophile susceptibility
- Separate assay for each drug type
- Affected by hematocrit
- Require pretreatment

Hamwi, et al. Cyclosporine Metabolism in Patients after Kidney, Bone Marrow, Heart-Lung, and Liver Transplants in Early and late Posttransplant Periods. Am J Clin Pathol 2000;114:536-543

Transplant Type
A=Kidney
B=Bone Marrow
C=Heart-Lung
D=Liver



White bars = Early period (≤ 3 months)
Black bars = Late period (>3 months)

We Needed a Killer App!

- Measure Rapamycin
- Short TAT / same day results
- Moving manual procedures out of core lab
- High accuracy for parent drugs
- Multiplexed if possible

2003: What Were Other Labs Doing for IS Drugs?

Reference Laboratories	Methodology
Quest	HPLC and TDx (by request)
Mayo Labs	HPLC + LC- MS/MS
LabCorp	LC- MS/MS
ARUP	LC - MS/MS

Academic Centers	Methodology
MUSC	LC - MS/MS
UNC	LC - MS/MS
Univ. of Mich.	LC - MS/MS
Univ. Penn	HPLC (LC - MS for C2 protocols)
Univ. Washington (Seattle) Children's	TDX
Duke	LC - MS/MS

LC-MS/MS Analyses

- Most methods are *home brews*
  - Resources:
    - Develop from scratch
    - Previously validated method from colleagues
    - Literature
    - Vendors
    - ***Most variation between labs related to differences in methods & standards***
- CLIA – High Complexity
- Highly specific for parent compounds
- Capable of being multiplexed

LC-MS/MS Proposal

Measurand	Volume / yr	Cost* / Analysis	Cost / yr
Cyclosporine	7588	$6.35	$48,184
Tacrolimus	13120	$6.35	$83,312
Rapamycin	744	$6.35	$4,724
TOTALS	**21,452**		**$136,220**

* Cost includes reagents, labor, & instr. depreciation

- All drug TATs within 1 day
- Capable of expanding to meet increased need
- Runs are multiplexed
- Total cost to system over 7 years = $953,540
- Saving to system over 7 years = $1,021,391
- Provides opportunities to do other assays

Process for Developing a Clinical Method

Make an Implementation Plan

- Method Selection
- Selection of Key Operator
- Outline/plan methods
- Acquisition of materials
- Set quality goals
- Validation
- Monitoring and statistics
- SOP preparation, staff training

Validate the Method

- Imprecision
- Recovery
- Linearity
- Sensitivity
- Specificity
- Interferences
- Specimen
- Method Comparison
- Assay Calibration

Guidance Documents

- EU Directive 2002/657/EC
- FDA Guidance 118
- FDA Special Controls Guidance for Particular Drugs
- CAP Chemistry & Toxicology Checklist
- CLSI Guidance EP10-A3E
- SOFT and AAFS Guidelines
- WADA Identification Criteria
- New York State – Clinical Laboratory Standards of Practice

EP50 Mass Spectrometry in the Clinical Laboratory

- General overview of mass spectrometry and clinical applications
- General guidelines on analytical method development and validation
- Seeks to harmonize some of the international documents
- http://www.clsi.org/source/orders/free/C50-A.pdf

Two New CLSI Documents in the Works

- C-57 Mass Spectrometry for Androgen and Estrogen Measurements in Serum
- C-60 Liquid Chromatography / Mass Spectrometry Methods.
- Should be available by end of year or early 2014.

Issues Unique to LC-MS/MS Analyses

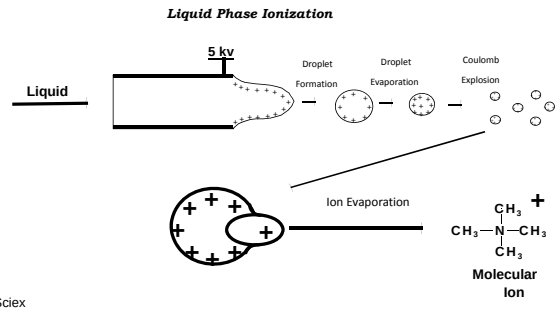
- New Terminology
- Matrix Effects
- Differential ionization of Internal Standards or Calibrators
- In Source Transformation (fragmentation)
- Isobaric Compounds and Isomers
- Cross-Talk effects
- Chromatographic Resolution
- Carry-Over

Vogeser & Seger, 2010, Clin Chem
http://www.clinchem.org/cgi/doi/10.1373/clinchem.2009.138602

Analytical Sensitivity

- **Limit of Absence (LOA)**
  - 20 replicates of zero calibrator or specimens without analyte run across multiple days
    - LOA = mean + 2SD or + 3 SD
- **Limit of Detection**
  - Measure specimens with levels (natural, diluted or spiked) that approximate the LOA, but are consistently detectable
    - LOD = mean of detectable concentration + 2SD or +3 SD of specimens
    - Also, noise x 3
- **Limit of Quantification (Functional Sensitivity)**
  - Minimum concentration where concentration can be measured reliably
    - Imprecision CV < 20%
    - Noise x 10
- **Goals: Must be below clinical needs**

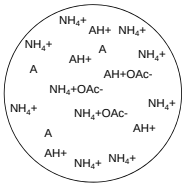
Theory of Electrospray (LC-MS)



Sciex

Effect of Matrix on Analyte Response in ESI

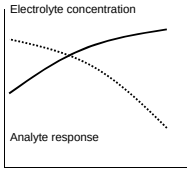
1. Surface competition



2. Charge competition

$OAc^- + AH^+ \rightleftharpoons HOAc + A$

Competition between analyte and electrolyte ions for conversion to gas-phase ions decreases analyte response.



Sample Preparation

- Matrix: Serum, whole blood, urine
- Pretreatment process
  - Protein Precipitation Protocols: Fast & easy, but associated with longer periods of ion suppression due to early eluting, low molecular weight matrix constituents.
  - Solid-Phase Extraction & Liquid-Liquid Extraction: Slower & more chance for error. Do have shorter periods of ion suppression.

Vogeser & Seger, 2010, Clin Chem
http://www.clinchem.org/cgi/doi/10.1373/clinchem.2009.138602

Matrix effects

1. Prepare extracts on 5 random blood samples from patients not receiving an IS drug.
2. Prepare 2 extracts using water as matrix.
3. To 500 uL of each extract add 150 ng Cyclo A, 75 ng Cylo D, 15 ng FK-506, 15 ng Rapa, and 20 ng Asco (all in 50ul).
4. Compare whole blood recoveries from water.

	FK-506 (Area)	Cyclo A (Area)	Rapa (Area)	Asco (Area)	Cyclo D (Area)
Water	**3651**	**75885**	**1267**	**8578**	**36918**
#1	4241	77293	1580	8650	38890
#2	4834	71217	1317	8787	34821
#3	4486	73005	1359	8218	27974
#4	4836	71500	1360	9023	36014
#5	4394	75963	1402	8225	32160
Blood mean	4559	73796	1404	8581	33972
Recovery	125%	97%	111%	100%	92%

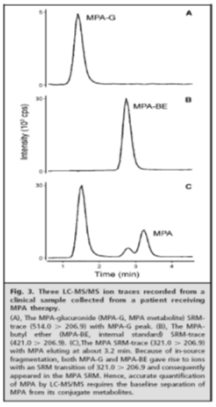


Fig. 3. Three LC-MS/MS ion traces recorded from a clinical sample collected from a patient receiving MPA therapy. (A), The MPA-glucuronide (MPA-G, MPA metabolite) SRM-trace (514.0 > 206.9) with MPA-G peak. (B), The MPA-butyl ether (MPA-BE, internal standard) SRM-trace (421.0 > 206.9). (C), The MPA SRM-trace (321.0 > 206.9) with MPA eluting at about 3.2 min. Because of in-source fragmentation, both MPA-G and MPA-BE gave rise to ions with an SRM transition of 321.0 > 206.9 and consequently appeared in the MPA SRM. Hence, accurate quantification of MPA by LC-MS/MS requires the baseline separation of MPA from its conjugate metabolites.

In Source Transformation:

- Fragmentation occurring after column separation but before collision cell
  - MAP-G > MPA > MPA Fragment
  - MPA-BE > MPA > MPA Fragment
- Can be a problem with endogenous drug metabolites
  - Check real patient samples in extended chromatographic runs

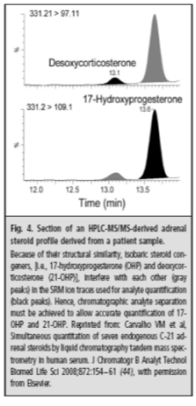


Fig. 4. Section of an HPLC-MS/MS-derived adrenal steroid profile derived from a patient sample. Because of their structural similarity, isobaric steroid congeners, [i.e., 17-hydroxyprogesterone (OHP) and deoxycorticosterone (21-DHP)], interfere with each other (gray peaks) in the SRM ion traces used for analyte quantification (black peaks). Hence, chromatographic analyte separation must be achieved to allow accurate quantification of 17-OHP and 21-DHP. Reprinted from: Carvalho VM et al, Simultaneous quantitation of seven endogenous C-21 steroid steroids by liquid chromatography tandem mass spectrometry in human serum. J Chromatogr B Analyt Technol Biomed Life Sci 2008;872:154–61 (44), with permission from Elsevier.

Isobaric Compounds & Isomers

- Extremely important when measuring endogenous analytes
- Mandates complete chromatographic resolution

Cross-Talk

- Occurs when several mass transitions with identical product ions are acquired over a short time interval
- If collision cell does not empty completely, spurious signals can be recorded in a subsequent trace
- Common when several metabolites of a single drug are detected with identical fragment ions
- Solution: Increase interscan delay

"Analyte co-elution is generally feasible due to the high selectivity of SRM/MRM experiments, if co-elution of isobaric analyte isomers or ion source fragmentation of the analyte metabolites can be ruled out"

Vogeser & Seger, 2008, Clin Biochem, 41:649-662

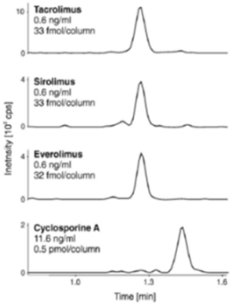


Fig. 4. Selected reaction monitoring (SRM) based ion trace chromatograms of a lower limit of quantification (LLOQ) standard of a routine on-line-SPE–HPLC–MS/MS method for immunosuppressive drug quantification [13]. The LLOQ (expressed in ng analyte/mL whole blood) was established well below the anticipated therapeutic ranges. A sensitivity in the lower fmol (analyte on-column) range can be easily achieved with the used high end mass spectrometer (API 4000QTrap operated in the SRM mode). Analyte co-elution is generally feasible due to the high selectivity of SRM/MRM experiments, if co-elution of isobaric analyte isomers or ion source fragmentation of analyte metabolites can be ruled out.

Carryover

The AOI at 500 ng/mL causes a 331% Carry-Over.

	10 ng/mL	100 ng/mL	500 ng/mL	
Plasma 1	537	451		517
Plasma 2	539	481		410
Analyte	108000	1010000	4700000	
Plasma 3	529	737		1660
Plasma 4	495	460		481
Analyte	107000	978000	4660000	
Plasma 5	464	831		1980
Plasma 6	522	581		528
Analyte	105000	985000	4550000	
Plasma 7	445	916		2900
Plasma 8	425	539		575

%Carryover = $(A_{avg} - B_{avg}) / B_{avg} \times 100$

A = plasma pool 3, 5 ,7 B = plasma pool 2, 4, 6, 8.

Immunosuppressant Drugs by LC/MS/MS – Protocol (circa 2004)

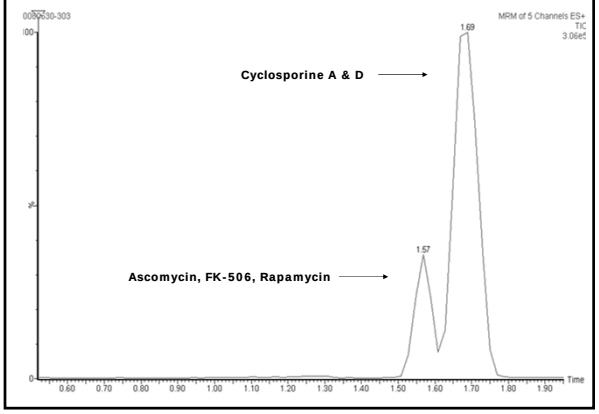
Microfuge tube protocol.

1. *In a 1.5mL Eppendorf tube accurately pipette:*
   50µL Lysis Solution A (0.4M $ZnSO_4$)
   200µL whole blood (Calibrators, QCs or patient samples)
2. Briefly vortex mix samples (5-10 sec)
3. Add 500µL of Precipitating Solution (25ng/mL Ascomycin + 100 ng/mL CycloD in acetonitrile)
4. Vortex mix for approximately 1 minute or until the entire sample is thoroughly mixed
5. Centrifuge for 5 minutes at 14,500rpm
6. Cut top off of micro-centrifuge vial. Place vial into HPLC autosampler
7. Inject 10µL on the LC-MS/MS system. Use C-18 column (4X3mm – Phenemonex)

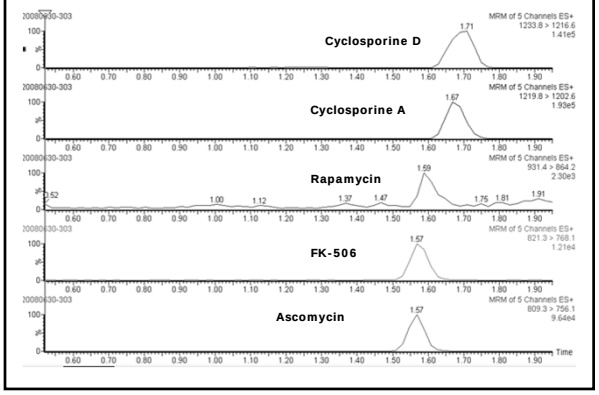
Immunosuppressant Drugs by LC/MS/MS - Protocol

Compound	Precursor Ion	Daughter Ion
Cyclosporine A	1220	1203
Tacrolimus	821.5	768.5
Rapamycin	931.6	864.5
Ascomycin	809.5	756.4
Cyclosporine D	1234.1	1217.2

Immunosuppressant Standard (20/20/500 ng/mL)



Immunosuppressant Standard (20/20/500 ng/mL)



Immunosuppressant Drugs by LC/MS/MS - Limits

Compound	LOD (ng/mL)	LOQ (ng/mL)	Linearity (ng/mL)
Tacrolimus	0.1	0.6	40
Cyclosporine A	3.0	10	1000
Rapamycin	0.3	0.6	40

Immunosuppressant Drugs by LC/MS/MS - Imprecision

Interassay			
	Tacrolimus	Cyclosporine A	Rapamycin
Concentration (ng/mL)	4	90	4
%CV	7	10	13
Intra-assay			
%CV	2	7	9

AMR & CRR

	AMR (ng/mL)	CRR (ng/mL)
Cyclosporine A	**100 - 1000**	**10 - 2000**
FK-506	**0.3 - 40**	**0.3 - 80**
Rapamycin	**0.6 - 40**	**0.6 - 80**

LC/MS/MS - Throughput

- Run time = 2 mins/sample
- Cycle time = 3.5 mins.
- One run contains 18 samples + 4 stds + 3 controls.
- A run takes 87.5 mins.
- 5 runs can be performed in 7.3 hours.
- This means a maximum of 90 specimens can be analyzed per shift per instrument.

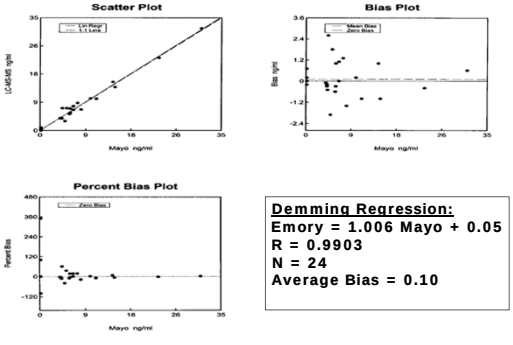
Emory Medical Laboratories Immunosuppressant Monitoring

- **Average monthly workload for 2004:**
  - **» Tacrolimus – 1322**
  - **» Cyclosporine A – 732**
  - **» Sirolimus – 426**

This equals 2480 samples a month or 83 samples per day

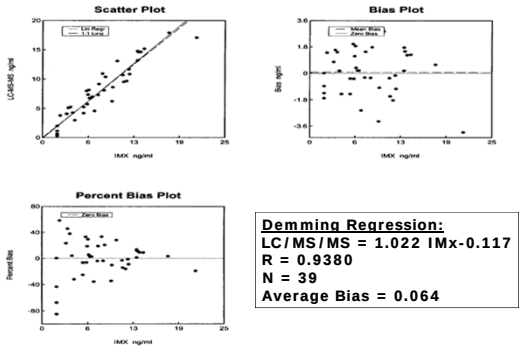
- **Analysis performed in the Special Chemistry Section, 7 days per week, dayshift only.**

RAPA – Assay Comparison (both LC/MS/MS)



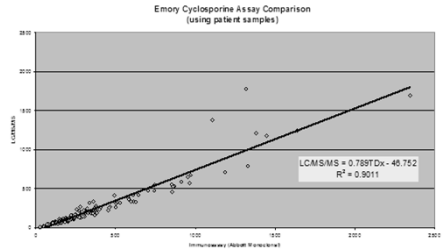
Demming Regression:
Emory = 1.006 Mayo + 0.05
R = 0.9903
N = 24
Average Bias = 0.10

FK-506 Assay Comparisons



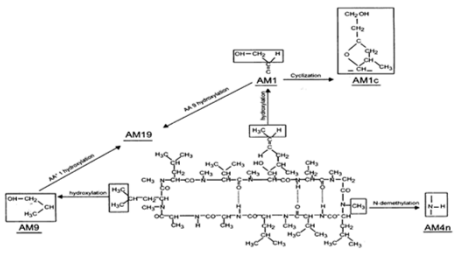
Demming Regression:
LC/MS/MS = 1.022 IMx-0.117
R = 0.9380
N = 39
Average Bias = 0.064

Patient Sample Comparison



n=185 patient samples with complete data as of 12/15/03
Therapeutic Range comparable to TDx assay: ≈ -7 to 269ng/mL
On average the LC/MS/MS values are 44% lower than the immunoassay values

Metabolic Pathway of Cyclosporine A



AM1, AM9, AM4n are the primary metabolites found in human blood and urine
Other metabolites result from further metabolism of the primary metabolites.

CsA Immunoassay today

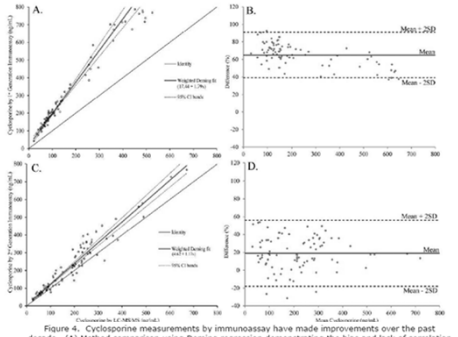


Figure 4. Cyclosporine measurements by immunoassay have made improvements over the past decade. (A) Method comparison using Deming regression demonstrating the bias and lack of correlation between the previous generation immunoassay and LC-MS/MS in 2004. (C) Data gathered in 2013 show the current immunoassay has improved agreement with LC-MS/MS. (B and D) Bland-Altman plots demonstrate an evident, yet decreased, positive bias in measurements when using an immunoassay versus LC-MS/MS for cyclosporine from years 2004 to 2013.

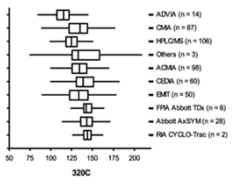
Molinaro R , Jour MS. Submitted for pub.

Cross Reactivity of Cyclosporine Major Metabolites

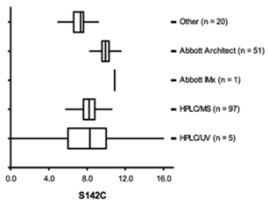
Metabolite	Bioactivity (%)	Concentration Relative to Parent (%)	Seimens ACMIA	Seimens ADVIA	Microgenics CEDIA	Roche EMIT	Syva EMIT	Abbott MEIA
AM1 (M17) 1-β-hydroxyl	10–20	80–150	1.8% @ 1000 ng/mL	<5% @ 1000 ng/mL	4.4% @ 1000 ng/mL	<AS @ 500 ng/mL	<0.3% @ 500 ng/mL - 5.9% @ 1000 ng/mL	-0.7–1.7% @ 1000 ng/mL
AM 9 (M1) 9-γ-hydroxyl	5–10	50–75	2.1% @ 1000 ng/mL	15% @ 1000 ng/mL	20.0% @ 1000 ng/mL	13% @ 667 ng/mL	7.3% @ 670 ng/mL 5.5% @ 1000 ng/mL	-3.8–1.9% @ 1000 ng/mL
AM 4N (M21) 4-N-demethyl	3–5	5–25	6.0% @ 1000 ng/mL	<5% @ 1000 ng/mL	16.0% @ 1000 ng/mL	5.1% @ 500 ng/mL	<0.3% @ 500 ng/mL 0.9% @ 1000 ng/mL	-2.3–3.1% @ 1000 ng/mL

Maynard S. Clin Lab News 2011; 36(8)

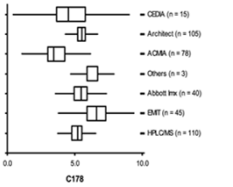
Cyclosporine (Bone Marrow)



Sirolimus (Liver)



Tacrolimus (Liver)



Figures show the inter-institutional precision of each method and the number of participants by method.

Source: Maynard S. Clin Lab News 2011;36(8) & Analytical Services International.

Common Problems

- Whole blood assays require extraction.
- Need for Standard Reference Materials (SRM) and Clinical Reference Materials (CRM)
- Reference methods are also needed to harmonize methods
- Lack of full automation

Extraction & Connectivity

- Current manual extractions are similar for both IA and LC-MS/MS
- Run Times:
  - IA = 12 to 18 mins/sample
  - LC-MS/MS = 2 to 4 mins/sample
- Most systems now accept bar code from sample labels to build work lists.
- Interfaces to connect to lab computer systems now available on many instruments.

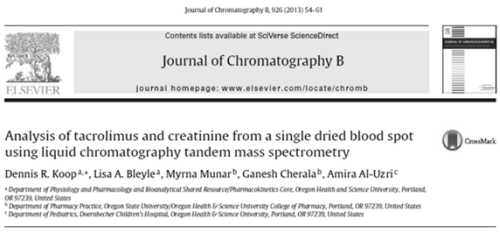
Standardization & Harmonization

- Standards:
  - Each IA manufacturer prepares their own
  - Many LC-MS/MS users prepare their own
  - Commercial STDS are available from 3 manufacturers
- Procedures:
  - Each IA vendor has specific extraction procedure(s), antibodies, buffers, & incubations
  - Most LC-MS/MS procedures are LDTs using different volumes, extractions, ionization modes, etc.

Trends in Immunosuppressant Monitoring

- Decrease dose of IS drug to as low as possible to maintain graft
- Most of current IS drugs exhibit kidney cytotoxicity to some degree
- Discontinuing use of Cyclosporine A
- Rapamycin used sparingly
- IS drugs are finding expanded use in autoimmune diseases.
- Should we be monitoring these patients?

Analysis from Dried Blood Spots



- Uses ~30 µL of whole blood (1 spot)
- Dried cards are stable for four weeks
- Cards could be mailed in
- Would allow for more frequent sampling & better dosing

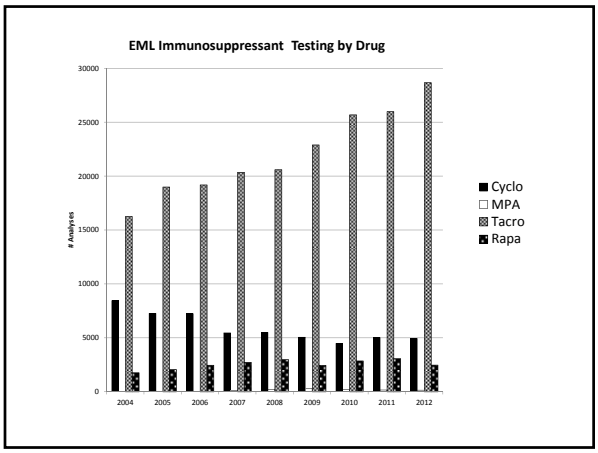
Conclusions

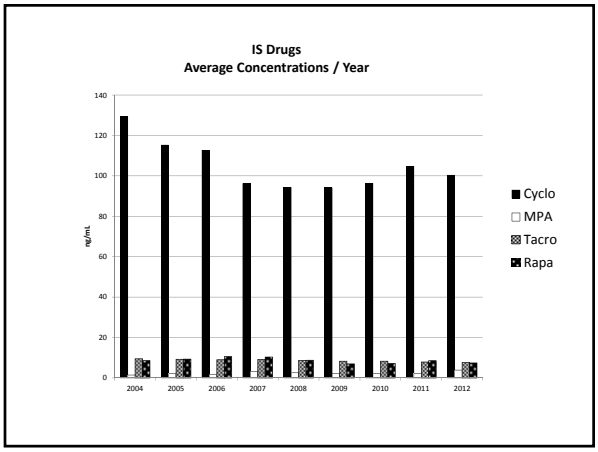
A. Both IAs & LC-MS methods can be used for TDM of IS drugs

B. LC-MS methods offer advantages of specificity and multiplexing

C. SRM, CRMs and a Certified Reference Method are sorely needed for each of the IS drugs routinely measured no matter what analytical technique is used.

D. Caution must be observed when comparing results between laboratories measuring IS drugs due to method differences





Status of LC-MS/MS Assays at EML Today:

Clinically in Use

- Cyclosporine
- Rapamycin
- Tacrolimus
- Mycophenolic acid
- Everolimus
- Busulfan
- Antidepressants (14)
- Antipsychotics (12)
- 25 (OH) Vitamin D
- Testosterone

Research

- Argatroban
- Lenalidomide
- Levamisole
- Bile Acids
- Iodothalomate
- Flumazenil

In Development

- Glucocorticoids
- Metanephrines
- Benzodiazepines
- Pain Medications

Thank you!!

Questions ???
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